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Convergence of multiple markers and analysis methods defines the genetic distinctiveness of cryptic pitvipers

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Abbreviated Title: Cryptic species delimitation using multiple markers and analysis methods.

Abstract

Using multiple markers and multiple analytical approaches is critical for establishing species boundaries reliably, especially so in the case of cryptic species. Despite development of new and powerful analytical methods, most studies continue to adopt a few, with the choice often being subjective. One such example is routine analysis of Amplified Fragment Length Polymorphism (AFLP) data using population genetic models despite disparity between method assumptions and data properties. The application of newly developed methods for analyzing this dominant marker may not be entirely clear in the context of species delimitation. In this study, we use AFLPs and mtDNA to investigate cryptic speciation in the *Trimeresurus macrops* complex that belongs to a taxonomically difficult lineage of Asian pitvipers. We analyze AFLPs using population genetic, phylogenetic, multivariate statistical, and Bayes Factor Delimitation methods. A gene tree from three mtDNA markers provided additional evidence. Our results show that the inferences about species boundaries that can be derived from population genetic analysis of AFLPs have certain limitations. In contrast, four multivariate statistical analyses produced clear clusters that are consistent with each other, as well as with Bayes Factor Delimitation results, and with mtDNA and total evidence phylogenies. Furthermore, our results concur with allopatric distributions and patterns of variation in individual morphological characters previously identified in the three proposed species: *T. macrops sensu stricto*, *T. cardamomensis*, and *T. rubeus*. Our study provides evidence for reproductive isolation and genetic distinctiveness that define these taxa as full species. In addition, we re-emphasize the importance of examining congruence of results from multiple methods of AFLP analysis for inferring species diversity.

Keywords: AFLP; Dominant marker; Pitviper; Taxonomy; Population structure; *Trimeresurus macrops.*

1 Introduction

Current efforts to discover and delimit species are usually facilitated by DNA sequence-based evolutionary reconstructions. However, this can be biased by locus-specific evolutionary constraints and genomic non-representation. In the case of recently diverged species, short nuclear sequences are often phylogenetically uninformative (e.g. Bardeleben et al. 2005, Weisrock et al. 2010). Multilocus markers are therefore highly recommended and offer quantitative advantages and genome-wide coverage (Zhang and Hewitt 2003, Meyer and Paulay 2005, Brito and Edwards 2009, Dupuis et al. 2012, Leaché et al. 2014). Routine phylogenomic analysis is still constrained by issues such as differences in results across methods, the need to integrate evolutionary histories of multiple loci, the lack of guidelines for best practices, and extensive computational requirements (Song et al. 2012, Gatesy and Springer 2013, Dell'Ampio et al. 2014, Faria et al. 2014, Leaché et al. 2014). Nonetheless, new methods have been developed and high-throughput sequence analysis is gaining popularity in evolutionary and speciation research (Morin et al. 2010, Springer et al. 2012, McCormack 2013, Misof et al. 2014).

Alternatively, the use of non-sequence-based multilocus markers has also increased, often revealing surprisingly clear, fine-scale genetic structure undetected by morphology and sometimes even by mtDNA markers (Brown et al. 2007, Egger et al. 2007, Kingston et al. 2009, Meudt et al. 2009, Milá et al. 2010). Among these, Amplified Fragment Length Polymorphism (AFLP) (Vos et al. 1995) is a time-tested, cost-effective, and powerful technique requiring no sequence knowledge. AFLPs have continued to prove useful for resolving species-level taxonomy, recovering patterns of speciation, evolutionary histories and inter-relationships, inferring population structure and genetic diversity analyses in a wide-range of animal species, such as butterflies (Kronforst and Gilbert 2008, Quek et al.

2010), cichlids (Albertson et al. 1999), salamanders (Wooten et al. 2010), lizards (Ogden and Thorpe 2002), dolphins (Kingston et al. 2009), and pinnipeds (Dasmahapatra et al. 2009).

1.1 Dominant marker analysis

Usually, multilocus markers are analyzed using genetic clustering and diversity analysis methods. These are implemented in population genetics models using F-statistics calculations based on allele-frequencies. The uses of these methods with respect to co-dominant datasets have been evaluated in both spatial and non-spatial models (Latch et al. 2006, Chen et al. 2007, Frantz et al. 2009). AFLPs, however, are dominant markers and do not allow distinction between homozygous and heterozygous states of an allele. Therefore, AFLP analysis using allele frequency-based population genetics models requires several assumptions to be made. As this results in analytical limitations, it is highly recommended that multiple analysis methods are applied, and inferences are made with high confidence only when results show congruence across methods (Carstens et al. 2013). However, the majority of AFLP studies continue to apply population genetic methods and derive biological inferences with rare discussion of possible analytical biases (Hollingsworth and Ennos 2004, Bonin et al. 2007). One such example is the routine use of the popular, non-spatial clustering program, STRUCTURE that uses a Bayesian MCMC algorithm to infer K – the number of populations. Other such programs include TESS, GENECLUST, and GENELAND that perform Bayesian cluster analysis under spatial models (Guillot et al. 2005a, 2005b, François et al. 2006, Chen et al. 2007, Guillot 2008, Guillot et al. 2008).

The algorithm used in STRUCTURE accommodates dominant data by assuming the presence of recessive alleles at a subset of loci that provide partial information about diploid genotypes for the entire dataset (Falush et al. 2007). GENELAND uses geographic coordinates and identifies groups of individuals in Hardy-Weinberg Equilibrium (HWE)

(Guillot et al. 2005a, 2005b). GENELAND was upgraded to correct allele frequency estimates from dominant data by taking into account observed genotypes and estimating unknown genotypes using model-based MCMC simulations (Guillot and Santos 2010). However, both STRUCTURE and GENELAND still assume that AFLP null-alleles (i.e., band absences) are recessive alleles for allele frequency calculations and subsequent K estimation. Common assumptions that drive these analyses (such as HWE in a population and linkage disequilibrium between populations but not within populations) are conceptually not applicable to dominant data. The models and assumptions used for K estimation in STRUCTURE are less than straightforward and need to be used with caution as they could yield inaccurate results (Pritchard et al. 2000). Furthermore, the accuracy of K estimation using dominant datasets in GENELAND is lower than in co-dominant datasets (Guillot and Santos 2010). In some cases, tree-building analysis of AFLPs has performed better at cluster identification due to the absence of population genetics model assumptions (Meudt et al. 2009). A certain degree of uncertainty is therefore unavoidable when using population genetic models to estimate the number of K, to assign individuals to each K, and to assess the genetic structure of each K. Hence, the need for new and more appropriate methods for dominant marker analysis has been identified (Hollingsworth and Ennos 2004, Excoffier and Heckel 2006, Bonin et al. 2007, Meudt et al. 2009).

Multivariate methods (such as factor and cluster analysis, principal component analysis, Multi-Dimensional Scaling, Molecular Analysis of Variance) implemented outside the confines of population genetics models have been extensively used to analyze AFLPs. More recently, two tools that use a combination of multivariate procedures to analyze multilocus genetic data were developed. Discriminant Analysis of Principal Components (DAPC) was developed in *adegenet* (an R package) as a method for inferring genetic clusters and genetic diversity using dominant data (Jombart et al. 2010). Hausdorf and Hennig (2010)

developed *prabclus*, also an R package, for species delimitation and ordination-cluster analysis using both dominant and co-dominant datasets. Both *adegenet* and *prabclus*, have performed better than STRUCTURE in initial studies (Hausdorf and Hennig 2010, Jombart et al. 2010). These methods could be useful for cluster and population structure analyses and speciation research that employ dominant markers.

Finally, Leaché et al. (2014) developed a new approach for AFLP and SNP based species delimitation by adapting a method called Single Nucleotide Polymorphism and AFLP Phylogenies (SNAPP) (Bryant et al. 2012). SNAPP produces posterior probability distributions of allele frequency changes and allows species tree estimation without the need for gene tree reconstruction and integration (Bryant et al. 2012). Grummer et al. (2013) first developed sequence-based Bayes Factor Delimitation (BFD) to perform marginal likelihood estimations (MLE) and test multiple species delimitation hypotheses. Species delimitation models are tested at the same time as species tree estimation, forgoing the need to specify a guide species tree (Grummer et al. 2013). Leaché et al. (2014) modified this and developed SNAPP BFD for species delimitation using SNPs and AFLPs. SNAPP BFD is implemented using MLE path sampling analysis in version 2 of Bayesian Evolutionary Analysis Sampling Trees (BEAST) software (Drummond et al. 2012, Bouckaert et al. 2014).

1.2 The study group – *Trimeresurus (Trimeresurus) macrops*

Asian green pitvipers from the genus *Trimeresurus* (Serpentes: Crotalidae: Crotalinae) (Lacépède 1804) are well known for cryptic speciation (e.g. Malhotra and Thorpe 2000, Vogel et al. 2004, Malhotra and Thorpe 2004a). The genus was divided into several genera in 2004 (Malhotra and Thorpe 2004b), among which was *Cryptelytrops* (Cope 1860). Recently, as a result of new information on the type species of *Trimeresurus*, *Cryptelytrops* was shown to be a junior synonym of *Trimeresurus* (David et al. 2011). Therefore, the species placed in

147 *Cryptelytrops* by Malhotra and Thorpe (2004b) are now correctly placed within *Trimeresurus*,
148 whether defined in a broader sense (by subsuming Malhotra and Thorpe's proposed genera as
149 subgenera) or narrower sense (continuing to accept the existence of several well-defined,
150 ecologically, genetically, and morphologically diagnosable generic-level units within the
151 former larger genus).

152 *Trimeresurus macrops sensu lato (s.l.)* is distributed across Thailand, Laos, Cambodia,
153 and Viet Nam, and was shown to consist of three cryptic species with disjunct geographic
154 ranges in the highlands of Cambodia (Fig. 1), distinguished by variations in several
155 individual morphological characters corresponding to their allopatric distributions (Malhotra
156 et al. 2011a). The populations have been proposed as three distinct species: (i) *T. macrops*
157 *sensu stricto (s.s.)* found in Thailand, south & central Laos, and northeast Cambodia, (ii) *T.*
158 *cardamomensis* (Cardamom Mountains green pitviper), from southeast Thailand and the
159 Cardamom mountains of southwest Cambodia, and (iii) *T. rubeus* (Ruby-eyed green pitviper),
160 found in southern Viet Nam and eastern Cambodia (Malhotra et al. 2011a). The morphology
161 and species ranges for each of these putative species have been fully described (Malhotra et
162 al. 2011a). A multivariate morphometric analysis, however, was not completely successful in
163 separating the three species (Fig. A.1), possibly due to geographic variation within each of
164 the species. Thus, whether the three populations are genetically distinct lineages and are
165 reproductively isolated needs to be clarified to fully support their species status.

166 Here we use multiple genetic markers and analysis methodologies to investigate the
167 genetic distinctiveness of the three proposed species in the *T. macrops* complex. We use three
168 mtDNA markers and multilocus nuclear marker set from AFLPs. We employ eight methods
169 of AFLP analysis incorporating population genetic, phylogenetic, multivariate statistical, and
170 Bayes Factor Delimitation approaches to confirm species boundaries. We provide a

comprehensive description of methods and results, and discuss them within the framework of each method for a better understanding of AFLP analysis.

2 Materials and methods

2.1 Sampling scheme

All samples were obtained from field collections, museums and private collections. Geographic distribution of samples within putative species ranges is as shown in Fig.1. Each locality is represented by multiple specimens in most cases. A total of 39 samples were used for mtDNA sequence analysis, including 22 samples of *T. macrops s.s.*, 6 samples of *T. cardamomensis* and 7 samples of *T. rubeus*. Samples covered the geographic distribution of much of the known range of *T. macrops s. l.*, and many of these also had morphological data available. Two closely related species were also included: *T. venustus* (3 samples from South Thailand and West Malaysia); and *T. kanburiensis* (1 sample, the only one available in our collection).

For AFLP analysis, a total of 50 individuals were genotyped among which 34 were shared with mtDNA analysis. Putative species in *T. macrops s.l.* were represented by: *T. macrops s.s.* = 22 samples (20 shared with mtDNA analysis); *T. cardamomensis* = 6 (all shared with mtDNA analysis); and *T. rubeus* = 7 (six shared with mtDNA analysis). Additionally, 15 *T. venustus* were genotyped (three shared with mtDNA analysis). However, the single *T. kanburiensis* sample available could not be successfully genotyped due to very low DNA yield. Full sample details including museum voucher numbers are provided in Table A.1

2.2 Experimental methods

2.2.1 mtDNA amplification and sequencing

196 Liver or muscle tissue in 80% ethanol, clippings of ventral scales in 80% ethanol, or
197 blood obtained from the caudal vein preserved in 5% EDTA and SDS–Tris buffer (100mM
198 Tris, 3% SDS) were used. Whole genomic DNA was extracted using standard salt
199 precipitation protocols (Sambrook et al. 1989). Three mitochondrial genes, 12S rRNA (12S),
200 16S rRNA (16S), and NADH4 (ND4), were amplified (as described in Malhotra et al. 2011b),
201 cleaned with shrimp alkaline phosphatase and Exonuclease I (Werle et al. 1994), and
202 sequenced using dye-labelled terminators (ABI PRISM™ BigDye™ Terminator Cycle
203 Sequencing Ready Reaction Kit) on an ABI 3730XL automated sequencer.
204
205 ***2.2.2 AFLP Genotyping***
206 Genomic DNA was extracted using GenElute™ Mammalian Genomic DNA Miniprep
207 Kit (Sigma-Aldrich). Extracts were duplicated for six samples using the same tissue type, and
208 three samples using different tissue types, for repeatability tests, and negative controls
209 (lacking any tissue) were included to monitor contamination. Extract quality was checked on
210 1% Agarose-EtBr gels, DNA was quantified on a NanoDrop ND-1000 Spectrophotometer,
211 and corrected to 10 ngμl^{-1} using 0.1M TE.
212 AFLPs were generated following the general protocol from Whitlock et al. (2008), but
213 with specific modifications as follows. 100 ng DNA was used per sample and 6.9 µl
214 digestion-ligation mix (final concentrations: 1X TA buffer, 0.17 µgμl^{-1} bovine serum albumin,
215 0.059 Uμl^{-1} each of *Eco*RI and *Mse*I enzymes, 0.3X T4 ligase buffer, 0.03 Uμl^{-1} T4 DNA
216 ligase, 0.74 µM each of *Eco* and *Mse* adaptors with 3 µl d_2H_2O) was added to make up a final
217 volume of 16.9 µl. This was incubated at 16ºC for 16 hours in a preconditioned water bath in
218 ThermoFast® 96-well plates (ABgene) and diluted by a factor of 1:4 (i.e. to a final volume of
219 50 µl) with d_2H_2O.

Pre-selective and selective primer sequences are provided in Table A.2. Fluorophore labelling of selective primers at the 5' end was performed by Applied Biosystems® using 6FAM, VIC, and PET labels. Combinations of selective primers and fluorophore scheme are given in Table A.2. Pre-selective amplification (PA) reactions were performed using 1 µl diluted ligated product in 10 µl reactions (final concentrations: 1X PCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM each of pre-selective *Eco*RI and *Mse*I primers, 0.025 $U\mu l^{-1}$ Thermoprime *Taq*) with 4.15 µl d_2H_2O. Thermocycling parameters were initial warm-up at 94°C for 2 minutes, 20 cycles of denaturing at 94°C for 30 seconds, annealing at 56°C for one minute, extension at 72°C for 2 minutes, and a final extension of 72°C for 10 minutes and 20°C for 5 minutes. PA products were diluted 1:10, and 1 µl of each, along with 5 µl loading buffer, was run on 1.5% Agarose-EtBr gels. Successful PAs resulted in a smear across the whole range of a 500bp ladder. 1 µl of diluted PA product was used for selective amplification (SA) in10 µl reactions with final concentrations same as PA except the primers were replaced by fluorophores and reverse selective primer. Thermocycling parameters were initial warm-up at 94°C for 2 minutes, 12 cycles of denaturing at 94°C for 30 seconds, annealing at 65°C Δ-0.7°C/cycle for 30 seconds, extension at 72°C for 1 minute, 23 cycles of 94°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 1 minute, and a final extension of 72°C for 10 minutes and 20°C for 5 minutes. SA products were diluted at 1:100 and 1 µl from each primer pair was poolplexed in 10 µl formamide along with 0.5 µl GeneScan™ 500 LIZ® Size Standard and the samples were processed on an ABI 3130XL Genetic Analyzer.

2.3 AFLP peak scoring

AFLP profiles were visualized and processed in GeneMapper® Software v4.0, and samples with amplification problems for one or more markers were discarded. Several

automated and semi-automated AFLP scoring methods have been proposed in an effort to reduce time, error, and subjectivity of peak calling (reviewed in Meudt and Clarke (2007)). We used the semi-automated method proposed by Whitlock et al. (2008) but found that direct application of this method to raw or filtered data (using the specified phenotype-calling threshold) resulted in significant numbers of inaccurate peak-calling from unaccounted false peaks (artefacts, inter-dye pull-ups, shoulder peaks, saturation peaks), peak mobility, and clear peaks failing to get called. Therefore, the data was first checked by eye and corrected for peak mobility, false peaks and uncalled peaks. Mean Peak Height (MPH) was calculated at each locus, a locus-selection threshold of 100 relative fluorescence units was applied. A relative phenotype-calling threshold of 20% of MPH was applied, i.e., all peaks ≥ 20% MPH were marked as present (1) and peaks ≤ 20% MPH were marked absent (0). We found our method, albeit time-consuming, significantly enhanced genotyping accuracy. Repeatability was measured as the number of loci with corresponding band presences across duplicated samples compared to the total number of loci scored.

2.4 Data analysis

2.4.1 Bayesian phylogenetic analyses

2.4.1.1 mtDNA phylogeny The mtDNA dataset consisted of 1450 bp including 350 bp of 12S, 474 bp of 16S, and 626 bp of ND4. The dataset was partitioned into 12S, 16S, and first, second, and third codon positions for ND4. Models of sequence evolution were inferred in jModelTest 2.1 (Guindon and Gascuel 2003, Darriba et al. 2012) using the Akaike Information Criterion (Posada and Crandall 1998). Mixed-model Bayesian analysis was implemented in MrBayes 3.1 (Ronquist and Huelsenbeck 2003) using the following models: Generalized time-reversible with gamma-distributed rate variation (GTR+G) for 12S and first and second codon positions of ND4; and GTR for 16S and third codon position of ND4.

Viridovipera vogeli was used as outgroup. Four independent MCMC analyses of 15,000,000 cycles each (sampled every 3,000 generations) were performed with one cold chain and three heated chains. The first 25% of trees were discarded as burn-in and a 50% majority-rule consensus tree was constructed from combined post-burn-in trees. Trace plots of clade probabilities were viewed using AWTY (Wilgenbusch et al. 2004).

2.4.1.2 Total evidence and AFLP phylogenies Bayesian phylogenetic inference was performed on both AFLP and total evidence (mtDNA + AFLP) datasets. A complex Bayesian model has been developed specifically for AFLP evolution (Luo et al. 2007). However the lack of algorithms and extreme computational burden (40,000 times slower than restriction site model implemented in MrBayes), make its implementation impractical (Koopman et al. 2008). We therefore used MrBayes 3.1 under the standard restriction data model by setting the coding bias to 'noabsencesites' for AFLP data to correct data for unobserved all-absence sites (Ronquist and Huelsenbeck 2003). The total evidence dataset was partitioned into AFLP, 12S, 16S, and ND4 partitions and model parameters estimated for mtDNA genes (Posada 2008). Four independent MCMC analyses were performed with 3 million cycles (sampled every 1000 generations) using one cold chain and three heated chains. The first 300,000 and 500,000 runs were discarded as burnin for AFLP and total evidence datasets respectively. A consensus tree was derived from post-burnin trees using all compatible groups. Final 50% majority rule consensus trees were constructed and re-rooted using the basal clade from the mtDNA reconstruction in FigTree v1.1.2 (Rambaut and Drummond 2008).

2.4.2 Bayes Factor Delimitation

Bayes Factor species delimitation was performed using SNAPP BFD (Leaché et al. 2014) implemented in BEAST2 (Bouckaert et al. 2014). Five different speciation hypotheses

were tested by lumping putative species in *T. macrops s.l.* in several combinations. In each hypothesis, *T. venustus* was included as a separate species. Details of speciation models are as follows with lumping of putative species indicated in parentheses: Model A: *T. macrops s.s., T. cardamomensis, T. rubeus;* Model B: *(T. macrops s.s. + T. cardamomensis + T. rubeus);* Model C: *(T. macrops s.s.+ T. cardamomensis), T. rubeus;* Model D: *(T. macrops s.s. + T. rubeus), T. cardamomensis;* Model E: *T. macrops s.s., (T. cardamomensis + T. rubeus).* Marginal likelihood estimation for each model was performed by path sampling conducted in 48 steps. MCMC chain length of 100,000 with pre-burnin of 10,000 for each step was sufficient to establish stationarity. The strength of support for competing models was evaluated as per Leaché et al. (2014), using the Kass and Raftery (1995) framework.

2.4.3 Population genetics methods

2.4.3.1 Descriptive statistics Estimating genetic diversity from dominant data under non-HWE is possible by incorporating population specific inbreeding co-efficients (F_{is}) into calculations of diversity indices such as F_{st} (Yeh et al. 1997, Foll et al. 2008). Although F_{is} values from small populations (less than 10 individuals) could be unreasonable (Holsinger and Lewis 2007), the difficulty of estimating allele frequencies from small populations could be overcome by analyzing a large number of loci (Krauss 2000). Given that sample sizes for two of our putative species were small (six and seven individuals), we incorporated F_{is} values from ABC4F (Foll et al. 2008) into F_{st} calculations in POPGENE (Yeh et al. 1997) and also calculated F_{st} in the *f* - free model in HICKORY 1.1 (Holsinger and Lewis 2007). An AMOVA was performed in *GenAlex v6.3* (Peakall and Smouse 2006) to calculate % genetic variance and Φ_{PT} (a distance-based analog of F_{st}) of populations, based on 9999 random permutations.

2.4.3.2 Detection of outliers BayeScan v1.0 (Foll and Gaggiotti 2008) was used to test for outlier loci in AFLP data (Pérez-Figueroa et al. 2010). Model parameters were automatically estimated based on 10 pilot runs (length = 5,000), using default chain parameters (sample size = 5,000, thinning interval = 20, and additional burn-in = 50,000). Jefferey's scale of evidence was set to maximum (decisive) and loci with $\log_{10}$ (Bayes Factor) = 2.0 (corresponding to $p = 0.99$) were considered outliers.

2.4.3.3 Estimation of historical gene flow Historical gene flow (N_m) among populations was estimated indirectly by the average of effective number of migrants exchanged between populations in each generation. Crow and Aoki's correction was applied, i.e., $N_m = (1-F_{st})/4\ \alpha\ F_{st}$, where N_m = the number of migrants per generation, and correction factor $\alpha = [n/(n-1)]^2$ where n = number of populations (Crow and Aoki 1984).

2.4.3.4 STRUCTURE Cluster analysis for dominant data was implemented in the admixture model using correlated allele frequencies – a more accurate model for assigning individuals to closely related groups (Pritchard et al. 2000, Falush et al. 2003, Falush et al. 2007). Ten runs of 100,000 iterations each were performed with K ranging from 1 to 10, and burn-in of 10,000 iterations. Since estimating the probability of K, Pr(X|K), is computationally difficult, two *ad hoc* methods were used: Ln Pr(X|K), prescribed by Pritchard et al. (2000), and ΔK based on the second order rate of change of likelihood function with respect to K, proposed by Evanno et al. (2005). Ln Pr(X|K) and ΔK for each K were plotted using STRUCTURE HARVESTER v0.56.4 (Earl and vonHoldt 2012). Assignment tests were performed to obtain the accuracy of assignment of individuals to putative species by including prior population membership information for each sample, and setting K=3 and 4 based on ΔK and Ln Pr(X|K) plots. Q matrices of population membership from 10 replicates were permuted in the

GREEDY_OPTION of CLUMPP v1.1.2 for a mean permuted matrix (Jakobsson and Rosenberg 2007). Results were visualized in *Distruct* (Rosenberg 2004).

2.4.3.5 GENELAND Preliminary test runs of 200,000 iterations were used to check for appropriateness of correlated and uncorrelated allele frequency model assumptions under both spatial and non-spatial priors. Based on these results, four independent MCMC runs of 500,000 iterations each were performed using a spatial prior with coordinate uncertainty fixed at 1km, uncorrelated allele frequencies, minimum and maximum K fixed at 1 and 16, and a burnin of 50,000 generations. An additional run of 1 million iterations and burnin of 100,000 generations was also performed to check for differences in K estimation due to increase in number of iterations. We tested the influence of spatial priors by rerunning the analysis after swapping the geographic co-ordinates of sample B44 (whose cluster assignment was incongruent with that of STRUCTURE) with that of sample A144 from north Thailand.

2.4.4 Multivariate statistical approaches

2.4.4.1 Principal co-ordinate analysis Principal co-ordinate analysis (PCoA), also known as Classical Multidimensional Scaling, is a traditionally used cluster analysis that transforms distances among objects into similarity/dissimilarity matrix (Gower 2005). This matrix is used to position objects in a space of reduced dimensionality while retaining the relationships between them. We performed a PCoA in MVSP v3.13n using Gower General Similarity Co-efficient (Gower 1966, 1971) to derive the similarity index among pairs of taxa. The resulting principal co-ordinates were plotted to visualize taxon clusters.

2.4.4.2 Discriminant analysis of principal components Discriminant Analysis of Principal Components (DAPC), implemented in *adegenet* in R, uses the *find.clusters* function to perform a Principal Component Analysis (PCA) and estimate overall genetic variance. When groups (such as species) are unknown, a K-means clustering algorithm divides total variance into among-group and within-group components. We ran K-means clustering several times, allowing K to vary from 1 to 49 in the first instance. Optimal number of clusters/putative species was determined by the lowest Bayesian Information Criteria (BIC). Discriminant Analysis (DA) of Principal Components (PCs) then defines a model which finds groups that maximize among-group genetic variability and minimize within-group variability (Jombart et al. 2010). We retained as many principal components in the preliminary data transformation step as necessary to represent 75% of total genetic variation. The optimal number of PCs to obtain a robust discrimination is estimated in *optim.a.score,* and the quality of discrimination is indicated by *a.score* for each cluster. We performed 30 DAPC simulations in *optim.a.score* for each of the 10 PCs retained. A second PCA, with K allowed to vary from 1 to 10, was performed to better visualize BIC results. A DAPC was performed using appropriate number of PCs to maximize *a.scores* according to *optim.a.score* result.

2.4.4.3 Gaussian clustering In *prabclus,* a Non-Metric Multi-Dimensional Scaling (NMDS) is performed on a distance matrix to derive Euclidean variables of genetic dissimilarity between individuals. Jaccard distances (Jaccard 1908) between individuals were calculated from the binary matrix using the *prabinit* function. As with DAPC, BIC was used as an indicator to estimate the number of clusters/putative species. A Gaussian mixture model determined clusters of individuals corresponding to mixtures of normal distributions that account for variation in data. Ten permutations of NMDS were performed on the distance matrix by *kruskal* method in three dimensions using *prabclust* function. To visualize the

clusters, the clusters object, showing assignment of individuals to each cluster, was exported to *Rcmdr* (Fox 2005).

2.4.4.4 Hierarchical clustering with seriation Finally, AFLP band presence and absence was visualized in *PermutMatrix V1.9.3*, a package originally developed for gene expression analysis (Caraux and Pinloche 2005). A Euclidean distance matrix of dissimilarity was generated and samples & loci were clustered based on McQuitty's unsupervised hierarchical clustering with a multiple heuristic seriation rule. Row-wise (taxa) and column-wise (locus) enumeration was optimized to improve visualization.

3 Results

3.1 Bayesian phylogenetic analyses

3.1.1 mtDNA phylogeny Partitioned Bayesian mtDNA phylogeny showed *T. macrops s.l.* samples forming three distinct clades (Fig. 2a). Samples from Thailand, Laos, and northeast Cambodia were assigned to the nominate species *T. macrops sensu stricto (T. macrops s.s.)* since this clade included specimens from the type locality (Bangkok, Thailand). The second clade consists of samples from south-eastern provinces of Thailand and southwest Cambodia. Finally, samples from southern Viet Nam and eastern Cambodia formed a sister group to all above clades, and to *T. kanburiensis*. These relationships are strongly supported with posterior probabilities of 100%. Recognizing the second and the third clades as *T. macrops s.l.* would render the species polyphyletic. The three clades therefore represent *T. macrops s.s.*, *T. cardamomensis,* and *T. rubeus* respectively.

3.1.2 Total evidence phylogeny The total evidence phylogeny consisted of mtDNA sequences and AFLP genotypes. The AFLP data comprised of 298 polymorphic loci from

330 loci with a repeatability score of 97% (for both duplicates for a given sample and for different tissue types with different storage conditions for a given sample). The total evidence tree (Fig. 2b) was topologically well-resolved and had robust support values. The three clades in *T. macrops s.l.* were well-differentiated, with 100% support values at deeper nodes and mostly high support values at the tips.

3.1.3 AFLP phylogeny The Bayesian AFLP tree (Fig. A.2) was poorly resolved at deeper nodes, with low support values. *T. cardamomensis* and *T. venustus* were not distinct from specimens of *T. macrops s.s*.

3.2 Bayes Factor Delimitation

For each speciation hypothesis, marginal likelihood values from SNAPP BFD analysis are shown in Table 2. All models derived by lumping of the three putative species in *T. macrops s.l.* in various combinations were decisively rejected based on $2 \times \log_e BF > 10$. Model A consisting of four species, where *T. macrops s.s., T. cardamomensis,* and *T. rubeus* were hypothesized to be three distinct species, was the most favored model.

3.3 Population genetics methods of AFLP analysis

3.3.1 Genetic differentiation and historical gene flow

F_{st} was 0.5 on average in both POPGENE and HICKORY. The % variation within and among putative species from AMOVA was 46% and 54% respectively. Overall Φ_{PT} was 0.538 and Φ_{PT} between populations are given in Table 1. The % polymorphic loci for *T. macrops s.s., T. cardamomensis and T. rubeus* were 54%, 21% and 25% respectively. A plot of $\log_{10}$ (BF) against F_{st} in BayeScan showed no outliers indicating neutrality of loci (Fig. A.3). Estimated historical gene flow, $N_{m,}$ among populations was 0.14.

3.3.2 Non-spatial and spatial Bayesian MCMC cluster analysis

In STRUCTURE, Ln Pr(X|K) increased by large increments up to K=4, while the increase was small at K=5 and decreased after K=6 (Fig. A.4a). With Evanno's methods, ΔK clearly peaked at three populations (Fig. A.4b). A graph estimating population structure for K=4 (including *T. venustus*) is given in Fig. 3. The probability of each individual belonging to assigned species is given in Table A.3. The probabilities of individual assignments when K=3 (Evanno's method) were nearly all equal to 1.0.

In GENELAND, three clusters could be visualized from the posterior distribution. Maps of individual posterior probabilities of membership to each cluster are given in Fig. 4. All individuals from the *T. cardamomensis* cluster were assigned to *T. macrops s.s.* Posterior probabilities of cluster membership for individual samples are given in Table A.4. The sample switching experiment showed that probability of membership of sample B44 to *T. macrops s.s.* increased to p=0.693 and to *T. venustus* decreased to p=0.158. Probability of membership of sample A144 to *T. macrops s.s.* decreased to p=0.550 (from p=0.664) and to *T. venustus* increased to p=0.304 (from p=0.158).

3.4 Multivariate statistical analysis of AFLPs

PCoA showed that ~50% of total variation was explained by the first three axes. These accounted for 24%, 18% and 7% of observed variation. A scatterplot showed the three proposed species formerly within *T. macrops* to be well separated on axes 1 and 3 (Fig. 5a).

The DAPC scatterplot clearly demarcated the three clusters corresponding to the putative species, with *T. venustus* as the fourth cluster (Fig. 5b). Assignment of individuals to each cluster in the former *T. macrops* (Fig. A.5a) agreed completely with geographic

distributions and mtDNA clades. The *a.score* for each cluster was: *T. macrops s.s.*=0.15, *T. cardamomensis*=0.96, *T. rubeus*=0.97, and *T. venustus*=0.68.

In *prabclus* four species clusters (including *T. venustus*) were detected and clearly separated in dimensions 1 and 3 with 100% accuracy of individual assignment to respective clusters (Fig. 5c). No datapoints were classified as noise components in this analysis (Fraley and Raftery 1998, 2002, Hausdorf and Hennig 2010).

Euclidean distance based hierarchical clustering and seriation also showed three clusters within *T. macrops s.l.* A partial map of AFLP banding patterns and the clustering results are presented in the graphical abstract, and full results are provided in Fig. A.6. *T. macrops s.s.* and *T. cardamomensis* samples differentiated into two distinct clusters within a single large cluster, except for ambiguous placement of A144. The position of *T. venustus,* nested within *T. macrops s.l.*, was congruent with phylogenetic analyses.

4 Discussion

4.1 Overview

In the majority of cryptic speciation studies, morphological conservativeness, parallel and convergent evolution of phenotypic traits, and/or mimicry (Sanders et al. 2006), driven by natural selection, sexual selection, and ecological adaptation, confound species delimitation. Southeast Asian green or “bamboo” pitvipers are typical in this sense due to their general morphological conservativeness or environmentally driven morphological convergence (Sanders et al. 2004). This is further complicated by sampling difficulties, due to cryptic life-styles of snakes, which can be a major drawback in population genetics analyses. In such cases, multiple markers and analysis methods may provide critical information to derive robust inferences on species diversity (Dupuis et al. 2012, Carstens 2013). Traditionally used in population genetics, AFLPs have recently found application in species

494 delimitation of a variety of plants (e.g. Prebble et al. 2012, Medrano et al. 2014) and animals
495 (e.g. Nie et al. 2012, Arthofer et al. 2013). Surprisingly, very few studies compare results
496 from multiple analysis methods (eg: Meudt et al. 2009, Reeves and Richards 2011). Most
497 comparative studies have only assessed genetic similarity co-efficients and multivariate
498 clustering methods such as UPGMA and NJ (Meyer et al. 2004, Kosman and Leonard 2005,
499 Dalirsefat et al. 2009).

500 In our investigation of cryptic speciation in *T. macrops*, we used three mtDNA
501 markers and 298 polymorphic AFLP loci and eight analysis methods. Of these, results from
502 two Bayesian phylogenetic analyses (total evidence and mtDNA), Bayes Factor Delimitation,
503 four multivariate statistical methods of AFLPs (PCoA, DAPC, Gaussian clustering, and
504 hierarchical clustering), and to a certain degree one population genetic method
505 (STRUCTURE) were congruent with each other. Moreover, these genetic results correspond
506 to allopatric geographic ranges and individual morphological characters described for each
507 species (Malhotra et al. 2011a). This gives us high confidence in the genetic distinctiveness of
508 *T. macrops s.s., T. cardamomensis*, and *T. rubeus* (Malhotra et al. 2011a).

509 In contrast, GENELAND was the only method that gave a contradictory result. This
510 brings into question the appropriateness of using GENELAND for a system such as this, and
511 we discuss this further in subsequent sections. The AFLP-only phylogenetic analysis, with
512 low support values, was unreliable. It is likely that low confidence can be placed in these two
513 results. We discuss our results and evaluate inferences in the context of dominant marker
514 analysis methods and provide a comparative assessment of phylogenetic, Bayes Factor
515 Delimitation, spatial and non-spatial Bayesian clustering methods, DAPC, Gaussian
516 clustering, and finally hierarchical clustering.

517

518 **4.2 Comparative assessment of results from AFLP analysis**

519 ***4.2.1 Phylogenetic analyses***

520 The resolution in total evidence Bayesian phylogenetic reconstruction matched that of
521 traditionally used mtDNA markers, producing a robust phylogeny with three distinct clades in
522 *T. macrops* (Fig. 2a & b). The AFLP tree, however, showed poor resolution of species and
523 low support values at deeper nodes (Fig. A.2). This difference in result indicates a stronger
524 phylogenetic signal from the mtDNA sequence data. AFLP-based phylogenetic analyses have
525 been largely confined to distance-based methods such as Neighbor-Joining and UPGMA in
526 the past. However, despite homology and non-independence of fragments, AFLP phylogenies
527 can successfully delimit species, sometimes performing better than STRUCTURE (Meudt et
528 al. 2009) and even mtDNA markers (Mendelson and Simons 2006, Kingston et al. 2009).
529 Combined AFLP and mtDNA datasets can yield robust phylogenies and provide evidence for
530 interspecific hybridization (Després et al. 2003, Pelser et al. 2003, Koopman 2005, Meudt
531 and Clarke 2007, Kingston et al. 2009). There have been conflicting reports on the utility of
532 AFLPs at deeper phylogenetic or interspecific levels as it appears to be affected by a drastic
533 increase in non-homologous shared fragments resulting in loss of phylogenetic signal
534 (Althoff et al. 2007, Dasmahapatra et al. 2009, Graves 2009, Kingston et al. 2009, García-
535 Pereira et al. 2014). Moreover, choice of bands and tree-building methods, and application of
536 restriction sites models, could over-simplify complex evolutionary processes, thus affecting
537 resolution of deeper nodes (Dasmahapatra et al. 2009, Graves 2009). Finally, the more
538 complex model developed specifically for AFLPs (Luo et al. 2007) has been found to be
539 extremely computationally burdensome, making it impractical to implement (Koopman et al.
540 2008, Dasmahapatra et al. 2009). The failure of our AFLP reconstruction under a restriction
541 site model reinforces the need for better, practical, and exclusive phylogenetic methods
542 (Graves 2009).
543

4.2.2 Bayes Factor Delimitation

SNAPP-BFD offers the advantage of testing multiple species hypothesis by integrating topologies during marginal likelihood estimation and thereby avoiding the need to predefine the species tree and biasing support (Leaché et al. 2014). From our SNAPP-BFD analysis, Bayes Factors ranked the four species model (three from *T. macrops* complex and *T. venustus*) as the most highly favored model (Marginal Likelihood=-3757) (Table 2). Model E, where we lumped *T. macrops s.s., T. cardamomensis and T. rubeus* into a single species, received the lowest support (Marginal Likelihood = -4587; 2x $\log_e$ Bayes Factor = 1660) and was ranked last among the five models tested. Therefore, the hypotheses that consider *T. macrops s.l.* as a single species, or as consisting of two species, are not supported by SNAPP-BFD analysis.

4.2.3 Non-spatial and spatial Bayesian MCMC clustering analyses

Both STRUCTURE and GENELAND use Bayesian MCMC methods to assign individuals probabilistically to populations based on allele frequencies. They cluster groups of individuals into populations by assuming that they are in HWE and linkage equilibrium. In the estimation of number of clusters (K), both STRUCTURE and GENELAND failed to differentiate *T. cardamomensis* as a separate species cluster and returned K = 3 by grouping these specimens in a single cluster with *T. macrops s.s.* (Fig. 3 & 4).

In STRUCTURE, the Pritchard et. al. (2000) method was inconclusive as to whether K equalled 3, 4, or even 5, as results depended on what cut off was applied to the Ln Pr(X|K) increase (Fig A.4a). In contrast, Evanno's method gave us a clear result of K = 3 (Fig. A.4b). Applying Evanno's correction is the norm for K estimation, since it is more formal and is endorsed by Pritchard et al. (2007). However, the Pritchard et. al. (2000) method of K estimation, which is said to be unreliable, subjective, and sometimes biologically

meaningless, proved more realistic when the Ln Pr(X|K) values were compared. The Ln Pr(X|K) increase from K = 3 to 4 was 8.57%, approximately 50% of increase from both K = 1 to 2 and K = 2 to 3, whereas it dropped to 2.1% for K = 4 to 5 (Table A.5). Moreover, there are several cases of K underestimation, and STRUCTURE results are said to be conservative when Evanno's method is applied (e.g., Frantz et al. 2009, Blanquer and Uriz 2010). Therefore, based on our phylogenetic results and taking into consideration that K = 4 proved a better estimation in the Pritchard et al. (2000) method of K estimation, we calculated the individual assignment probabilities by assigning individuals in *T. macrops s.l.* to three clusters and *T. venustus* individuals to a fourth cluster. The mean permuted assignment probability value *(p)* for *T. cardamomensis* individuals to a separate cluster (as per CLUMPP) was only slightly lower at $p = 0.969$ compared to *T. macrops s.s* ($p = 1.0$), *T. rubeus* ($p = 0.999$), and *T. venustus* ($p = 0.999$). This further increased our confidence that Evanno's method underestimated K by grouping *T. cardamomensis* with *T. macrops s.s.*

In GENELAND, however, all individuals of *T. cardamomensis* as well as *T. macrops s.s.* were assigned to *T. macrops s.s.* with a probability of assignment $p = 0.644$. Among the *T. macrops s.s.* samples, specimen B44, reportedly from Nakhon Si Thammarat in southern Thailand, was an exception with a lower probability of assignment to *T. macrops s.s* ($p = 0.441$). Moreover, while the probability of assignment of all other *T. macrops s.s.* specimens to *T. venustus* was $p = 0.158$, that of sample B44 was higher at $p = 0.363$. In contrast, the STRUCTURE analysis assigned B44 to *T. macrops s.s.* with a maximum mean permuted probability of 1.0 by CLUMPP. This specimen was the only *T. macrops s.s.* specimen reported from southern Thailand and as it was obtained from a dealer, the presence of this species at the reported locality is unconfirmed. Our suspicion, that this difference in assignment between GENELAND and STRUCTURE was due to the influence of spatial

priors on individual assignment probabilities in GENELAND, was supported by a sample switching analysis.

The apparent interruption of gene flow represented by the Gulf of Thailand, which currently separates populations in the south of Thailand from populations in Cambodia and Vietnam, might be considered to invalidate the GENELAND results. However, it is important to consider the context at the time these species were evolving. The Gulf of Thailand has a maximum depth of 80m, and Voris (2000) showed that for c. 35% of the last 170,000 years, it would have largely been dry land. While this precision is only possible for relatively recent timescales, it is also known that there have been sea level fluctuations of similar magnitudes during the last 30 my (Hall and Holloway, 1998).

Allele-frequency estimations from small populations could be compensated by analyzing large number of loci (Krauss 2000). Our AFLP dataset was large (298 loci), yet the genetic diversity indices show a clear bias caused by small sample sizes of *T. cardamomensis* and *T. rubeus*, since the % polymorphic loci decreased considerably relative to population size. F_{st} values were much lower for *T. cardamomensis* and *T. rubeus* (0.0507 and 0.00729, *p<0.00001*) as opposed to *T. macrops s.s.* and *T. venustus* (0.9, *p<0.00001*). However, Φ_{PT} values showed *T. cardamomensis* to be less genetically distinct from *T. macrops s.s.* than *T. rubeus* (Table 1). These estimates appear to be non-representative, with biases arising from sampling deficiency, probably insufficient number of loci, as well as dominant nature of the marker. Hence deriving any strong biological inferences from diversity indices would be highly dubious. Our estimation of historical gene flow among *T. macrops* complex populations was low (N_m=0.14), although N_m calculation is based on F_{st}. Moreover, considering the wider geographic distribution of *T. macrops* complex and geographical barriers in southeast Asia, it is likely that there is established allopatry among populations. Therefore, it seems highly likely that implementing GENELAND (which is more appropriate

for systems with contemporary gene flow) may not have been ideal for the *T. macrops* complex. It is difficult to predict whether increasing sample size may have provided better resolution for *T. cardamomensis* in STRUCTURE and GENELAND. *Trimeresurus rubeus* (represented by only seven individuals), was still sufficiently genetically diversified to form a separate cluster as well as achieve 100% individual assignment success in all analysis methods.

4.2.4 Multivariate statistical methods

Multivariate techniques proved superior to Bayesian MCMC clustering in terms of sensitivity and confidence as PCoA, DAPC, Gaussian clustering, and hierarchical clustering all split *T. macrops s.l.* into three clusters and also assigned individuals to their corresponding mtDNA clades with 100% success (Fig. 5a, b, & c and Fig. A.6).

In DAPC analysis, *optim.a.score* recognized that the first three PCs would give highest *a.scores*. Although they represented only 45% of total variance, we used the first three PCs to obtain a strong and stable DAPC solution (Thibaut Jombart, personal communication). *a.scores* were very high for *T. cardamomensis* and *T. rubeus* (0.96 and 0.97), and this increases our confidence that these two groups are genetically distinct. *T. macrops s.s.* had an *a. score* of 0.15, but given that some issues still exist with *optim.a.score* and *a.score* functions (e.g., with repeatability), a more critical review of these scores is not justified at this stage. Gaussian clustering in *prabclus* was highly successful, defining three clusters of *T. macrops* and assigning individuals accurately to them. It is important to note that both DAPC and Gaussian clustering were not affected by any sampling deficiency or by lower level of genetic diversity of *T. cardamomensis*, which was always identified as a separate cluster, in contrast to Bayesian clustering.

Finally, the Euclidean distance-based hierarchical clustering and seriation also showed distinct clustering of four species with *T. venustus* nested in *T. macrops s.l.. T. macrops s.s.* and *T. cardamomensis* each formed a distinct sub-cluster within a single large cluster (Fig. A.6). Locus-based (column-wise) clustering identified regions of banding dissimilarity across the three species in *T. macrops s.l.* (Graphical Abstract). Sample A144, which clustered with *T. cardamomensis* is the only representative from North Thailand (Jae Sorn NP, Lampang province) and likely a genetic outlier. It is interesting that this was only apparent in the hierarchical clustering analysis.

5 Conclusions

Our study provides the genetic evidence required to complete investigations into the morphologically cryptic species in the *Trimeresurus macrops* complex. Congruence of results between multiple markers and methodologies clearly demarcates the three proposed species as genetically and reproductively isolated. We therefore confirm *Trimeresurus macrops s.s., T. cardamomensis, and T. rubeus* as full species. Further, our study reinforces the importance of using appropriate and multiple analysis methods and performing a comparative assessment before deriving inferences on species diversity. The study also demonstrates the continued utility of AFLPs for cryptic species delimitation and discovery, when high cost and sequencing noise can be deterrents for using high-throughput sequencing (e.g. RAD-Seq) (Davey et al. 2013).

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FIGURE CAPTIONS

Figure 1. Map of southeast Asia showing sampling scheme for mtDNA and AFLP analysis of the three putative species formerly assigned to *Trimeresurus macrops*, plus closely related species *T. venustus and T. kanburiensis*. MtDNA analysis included 39 samples and AFLP genotyping included 50 samples, of which 34 were also used in mtDNA analysis. The datasets used for the samples are represented by: empty circles (mtDNA); solid circle (AFLP); solid circle inside empty circle (both mtDNA and AFLP). Each location may represent multiple overlapping samples. The distribution of species in *T. macrops s.l.* is based on Malhotra et al. (2011a) and represents: *T. macrops sensu stricto* (orange)*; T. cardamomensis* (blue)*;* and *T. rubeus* (red). Samples in clear represent *Trimeresurus kanburiensis* from west Thailand and *T. venustus* from southern Thailand and Malaysia.

Figure 2. Mixed-model Bayesian phylogenetic reconstructions showing multiple distinct clades in the *Trimeresurus macrops* complex. 50% majority-rule consensus phylograms with Bayesian posterior probabilities derived from a) mtDNA and b) Total evidence dataset. The phylogenies are highly congruent and show three distinct clades corresponding to *T. macrops sensu stricto*, *T. cardamomensis,* and *T. rubeus*.

Figure 3. Population structure of the *Trimeresurus macrops* complex and *T. venustus* estimated using STRUCTURE. Summary plot shows estimated population assignments based on K = 4 with each color corresponding to a species.

Figure 4. Map of posterior probabilities of species membership and spatial location of genetic discontinuities estimated in GENELAND. Three clusters (K = 3) could be

visualized from the posterior distributions. The three plots represent assignment of pixels to each cluster: a) *T. macrops sensu stricto* and *T. cardamomensis*; b) *T. rubeus*; and c) *T. venustus*. Lightest colours indicate highest probabilities of membership and contour lines represent the spatial position of genetic discontinuities between species.

Figure 5. Scatter plots from multivariate analyses showing four clusters representing three species in *T. macrops* s.l., and *T. venustus*. The four species clusters are represented by different symbols (circles: *T. macrops sensu stricto;* triangles: *T. cardamomensis*; vertical crosses: *T. rubeus*; and diagonal crosses: *T. venustus*).

a) PCoA. Eigenvectors 1 and 3 using Gower General Similarity Coefficients are plotted. b) DAPC. The first three PCs of the DAPC are plotted. c) *prabclus.* Gaussian clusters on dimensions 1 and 3 identified from Non-metric Multi-Dimensional Scaling

Table 1 Pairwise Φ_{PT} values for populations of *Trimeresurus macrops* complex and outgroup *T. venustus.* Φ_{PT} were derived from AMOVA of 298 AFLP markers using 9999 random permutations. Significant *p-values (p<0.05)* are highlighted by *.

Species	*T. macrops s.s.*	*T. cardamomensis*	*T. rubeus*	*T. venustus*
T. macrops s.s.				
T. cardamomensis	0.328*			
T. rubeus	0.555*	0.627*		
T. venustus	0.494*	0.579*	0.694*	

Table 2 Bayes Factor Delimitation of species in the *Trimeresurus macrops* complex using AFLPs (298 loci). Alternate species delimitation models were tested using SNAPP BFD against the four species model (Model A). Lumping of species is indicated by parentheses. The four species model is significantly better than all other models by 2 x $\log_e$ BF ≥ 408.

Model	Model Details	Species	ML	Rank	2 x $\log_e$ BF
A	*T. macrops s.s., T. cardamomensis, T. rubeus,* & *T. venustus*	4	-3757	1	
B	*(T. macrops s.s. + T. cardamomensis + T. rubeus)* & *T. venustus*	2	-4587	5	+1660
C	*(T. macrops s.s.+ T. cardamomensis), T. rubeus,* & *T. venustus*	3	-3961	2	+408
D	*(T. macrops s.s. + T. rubeus), T. cardamomensis,* & *T. venustus*	3	-4384	4	+1254
E	*T. macrops s.s., (T. cardamomensis + T. rubeus),* & *T. venustus*	3	-4138	3	+762

ML = Marginal likelihood
BF = Bayes factor